THE EFFECT OF INCREASING TIME OF DEVELOPMENT AT CONSTANT TEMPERATURE ON THE WING SIZE OF VESTIGIAL OF DROSOPHILA MELANOGASTER

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Introduction

This paper deals with the effect of nipagin (methyl parahydroxy benzoate) and poor food conditions on the wing size of the mutant vestigial in *D. melanogaster.* Nipagin is being used in many laboratories as an antiseptic for mold control in *Drosophila* culture media. It was found at this laboratory that the time of development of an isogenic stock was increased when the larvae were raised on nipagin-treated food. This suggested a method for increasing the time of development at constant temperature, a new tool in phenogenetic research.

The effect of temperature on the wing size of vestigial has been studied by a number of investigators (Harnly, 1930, 1932; Stanley, 1928, 1931, 1935; Hersh and Ward, 1932; Li and Tsui, 1936). They find that the wing size of vestigial increases with increasing temperature. Furthermore, the temperature-effective period occurs during a relatively short portion of the larval life. This present work represents a different approach to the problem in that it concerns the effect of prolonging the duration of the larval period at constant temperature.

Methods

The culture medium consisted of 850 cc. water, 100 grams cornmeal, 150 cc. molasses, 13 grams agar-agar, and 6 grams brewer's yeast, made up in the usual manner. The cultures were seeded with dry yeast. In the starvation experiments the dry yeast was not added. The nipagin was weighed on a standard quantitative balance and thoroughly mixed with the food before pouring. Half-pint milk bottles containing 60 cc. food were used.

The isogenic vestigial stock was obtained from Dr. A. Hersh of Western Reserve. About 20 pairs were used for egg-laying. It was found that the flies did not lay well on the food containing the higher concentrations of nipagin. It was necessary to use long egg-laying periods of twelve hours and for this reason the time of development was determined only approximately for these concentrations.

The egg-laying and total development was carried out in an incubator held constant at 28° ± 0.1°. The incubator was kept in a constant temperature (16° ± 1.°), constant humidity (60 per cent ± 5 per cent relative humidity) room. The apparatus is fully described in Drosophila Information Service 6, April 1936.

As the flies hatched they were examined, the sexes were separated and they were placed in vials containing 70 per cent alcohol. An unselected sample of control flies and 0.2 per cent nipagin flies were bred for a second generation to determine any "carry-over" effect. The wings of the flies were removed under a binocular microscope and

TABLE I

Effect of nipagin on wing size of vestigial. Temperature, 28°±0.1°.

Conc. of Nipagin in per cent	Time of Pupation (hours)	♀♀			♂♂		
		No.	Length in mm. ±s.e.	Area in mm.² ±s.e.	No.	Length in mm. ±s.e.	Area in mm.² ±s.e.
0.0	98	40	0.85 ±0.017	0.178 ±0.0047	14	0.76 ±0.023	0.132 ±0.0033
0.05	104	44	0.90 ±0.007	0.186 ±0.0043	25	0.91 ±0.035	0.177 ±0.0078
0.1	112	37	0.90 ±0.015	0.171 ±0.0044	41	0.95 ±0.024	0.189 ±0.0072
0.2	146	50	1.02 ±0.003	0.213 ±0.0094	45	1.06 ±0.035	0.221 ±0.0094
Carry-over effect							
F_1 from random sample of .2 per cent nipagin-treated flies							
0.00	103	31	0.95 ±0.023	0.206 ±0.0055	38	0.85 ±0.0075	0.167 ±0.0038

mounted on slides with a drop of cedar oil. The right wing was used unless it was torn or mutilated.

The wings were projected with a Proni projection apparatus. The magnification set at 75 × was checked periodically with a stage micrometer. The periphery of the wings were traced and from these tracings the maximum lengths and areas were measured. A Glogau vernier caliper and a Keuffel and Esser planimeter were used.

In the later experiments 0.1 per cent nipagin was used. It was suspected that nipagin produced its effect by slowing down the growth of yeast and thereby decreasing the food supply. To check this a number of cultures were prepared with no addition of live yeast. In these non-

seeded bottles a number of old larvae were added after the egg-laying. This was done to remove any yeast carried in on the bodies of the adult flies.

The time of development was determined by removing pupae at intervals of four hours and placing them on agar slants. Many of these flies were used for a second generation test. The matings were control × control, control ♂♂ × nipagin-treated ♀♀, control ♀♀ × nipagin-treated ♂♂ etc. to determine how much of the carry-over effect was maternal or paternal.

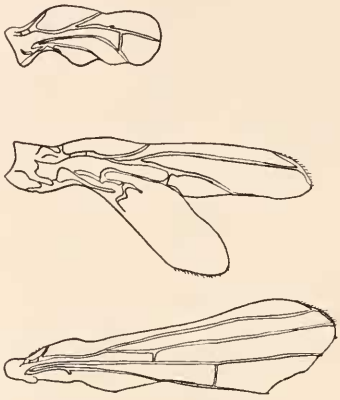

Fig. 1. A normal 28° vestigial wing compared with wings from 28° nipagin treated flies.

Experimental

The Effect of Varying Concentrations of Nipagin

In these experiments the larvae were raised on nipagin-treated food to determine the effect of nipagin on the time of development and wing area. The concentrations of nipagin used were 0.05 per cent, 0.1 per cent, 0.2 per cent, 0.4 per cent, and 0.8 per cent of the food weight. A few larvae in the 0.4 per cent and 0.8 per cent developed to pupation but failed to hatch. It was found that the time of development increased with increasing concentrations of nipagin, the 0.2 per cent pupating two days later than the controls.

Table I shows the wing length and area as affected by nipagin. With one exception, the 0.1 per cent for females, the lengths and areas of the vestigial wings increase with increasing concentrations of nipagin. The wing size of the control males is smaller than that of the females. With increasing concentrations of nipagin, however, the male wing size increases faster than the female. At 0.1 per cent and 0.2 per cent the

male wings are larger than the female. A similar result is obtained with temperature, the male wing size exceeding that of the females at high temperature (Harnly, 1930; Stanley, 1931).

It was found when preserving the flies at hatching, that with increasing time of development there was an apparent increase in wing size within each nipagin-treated population. The change in wing size due to nipagin is, therefore, greater than the means given in Table I; these means having been obtained by including all the flies in a given population irrespective of their time of development. This general result has been recently reported by Braun (1939) on notch.

TABLE II

Relation between time of pupation and wing area. Control series.

Time of Pupation in hours	♂♂		♀♀	
	No.	Area in mm.	No.	Area in mm.
1. 82	8	0.130	3	0.164
2. 86	6	0.138	6	0.137
3. 90	15	0.137	6	0.159
4. 94	25	0.136	10	0.143
5. 98	7	0.154	11	0.173
6. 102	3	0.158	4	0.147
7. 106	3	0.133	2	0.187
8. 110	0		2	0.167
9. 114	3	0.107	2	0.154
	mean time, 93.2±0.87 hours mean area, 0.139±0.004 sq. mm.		mean time, 96.1±1.24 hours mean area, 0.156±0.0035 sq. mm.	

Figure 1 illustrates the appearance of the larger wings obtained from a 0.2 per cent nipagin population as compared with a "normal" vestigial wing. The larger wings simulate the expression of other vg. alleles when raised under normal environmental conditions.

The results obtained by raising a random sample of 0.2 per cent nipagin-treated flies for another generation but in untreated food are shown in Table I. Both the mean length and area show a significant carry-over effect. Some of these data had been reported previously (Child and Albertowicz, 1936).

Effect of Time of Development

In the second series of experiments the relation between time of development and wing area was determined. Starvation and 0.1 per cent nipagin were used. The larvae were removed from the culture as

they pupated and the areas of the wings were determined separately for each pupating group. The results (Table II) indicate that in the control series there is no apparent effect of time of development (from egg-laying to pupation) on the size of the wings. The larvae pupate between 82 hours and 114 hours and the wing areas among the different groups do not differ significantly from one another.

The time of development is very markedly increased in the nipagin and starvation series (Tables III and IV). The wing areas of the various groups show greater differences than in the control series. There is an apparent relation between the time of pupation and wing

TABLE III

Relation between time of pupation and wing area after treatment with 0.1 per cent nipagin.

Time of Pupation in hours		♂♂		♀♀	
		No.	Area in mm.	No.	Area in mm.
1.	93	10	0.158	12	0.167
2.	97	8	0.231	5	0.181
3.	101	2	0.437	4	0.140
4.	107	23	0.299	12	0.172
5.	118	9	0.368	8	0.181
6.	129	5	0.592	5	0.236
7.	141	6	0.517	1	0.268
8.	153	1	0.505	3	0.233
9.	165	25	0.268	8	0.151
10.	179	23	0.267	23	0.165
11.	191	7	0.318	14	0.208
12.	203	12	0.379	14	0.190
13.	215	14	0.354	27	0.201
mean time, 152.4±3.39 hours mean area, 0.315±0.0113 sq. mm.				mean time, 163.5±3.81 hours mean area, 0.185±0.0044 sq. mm.	

area. This relation is more easily observed on the imagoes as they hatch. With increasing time of development the larvae (and the flies) become smaller and smaller so that the relative difference between wing area and body size is very great in the delayed flies although the absolute area increases and then decreases. Unfortunately the body size of the adults was not measured and we are unable to show this difference quantitatively.

The Carry-over Effect

The carry-over effect was studied using normal food. To determine whether both sperm and eggs from treated parents transmitted the fac-

TABLE IV

Relation between time of pupation and wing area after starvation.

Time of Pupation in hours	♂♂		♀♀	
	No.	Area in mm.	No.	Area in mm.
90	7	.204	5	0.190
94	3	.164	3	.310
98	2	.197	12	.185
102	5	.194	2	.238
106	1	.224	4	.188
110	2	.169	7	.169
116	17	.277	16	.201
122	4	.258	8	.196
126	4	.283	4	.215
130	3	.212	4	.100
138	6	.242	9	.300
150	0		3	.231
162	1	.345	1	.212
174	2	.642		
	mean time, 116.4±2.53 hours mean area, 0.254±0.0174 sq. mm.		mean time, 115.0±2.13 hours mean area, 0.213±0.0102 sq. mm.	

tors for increased wing size, treated males and females were mated with control females and males respectively. Treated males were also mated with treated females. The results of these various reciprocal crosses are shown in Table V which also includes the control areas and the means of Tables III and IV. The carry-over effects are more apparent

TABLE V

Effect of .1 per cent nipagin and starvation.

Experiment	♂♂			♀♀		
	Time of Pupation	No.	Area±s.e.	Time of Pupation	No.	Area±s.e.
	hours		*mm.²*	*hours*		*mm.²*
Control	93.2±0.87	70	0.139±.0040	96.1±1.24	48	0.156±.0035
.1% Nipagin	152.4±3.39	145	0.315±.0113	163.5±3.81	136	0.185±.0044
Starved	116.4±2.53	57	0.254±.0174	115.0±2.13	78	0.213±.0102
Previous Treatment	Carry-over effect F_1 from control and treated flies					
Control ♀ × Nipagin ♂	110	94	0.203±.0058	110	81	0.181±.0019
Control ♀ × Starved ♂	110	33	0.203±.0163	110	49	0.183±.0025
Nipagin ♀ × Control ♂	116	25	0.229±.0270	116	36	0.201±.0167
Starved ♀ × Control ♂	116	45	0.281±.0203	116	56	0.192±.0060
Nipagin ♀ × Nipagin ♂	116	40	0.279±.0215	116	44	0.174±.0042
Starved ♀ × Nipagin ♂	116	55	0.356±.0259	116	31	0.224±.0141
Control ♀ × Control ♂	95	22	0.143±.0052	95	19	0.161±.0037

in the male offspring than in the female offspring, since in the latter the total effect is smaller. The time of development is only approximate, not having been measured by pupa removal but by simply noting the time when about half the larvae had pupated. It is quite evident that in all of the matings the wing areas are greater when affected flies of either sex are used as parents. When treated females are used as parents the difference is greater than when males are used. Treated males and females as parents have offspring with greater wing areas than those obtained when only one treated parent is used. Starvation of the parents seems to produce a greater effect in the offspring than nipagin treatment.

Discussion

It is well known that with increasing temperature there is an increase in the wing size of vestigial. A sharp increase is not obtained, however, until very high temperatures are reached. It is generally accepted that temperature produces its effect by affecting differentially the rate or duration of the " vestigial reaction " as compared with the rate or duration of other developmental processes. By vestigial reaction we mean the developmental reaction or reactions in the vestigial fly which differ in rate or duration from the reactions in their isogenic wild type.

In the experiments with nipagin there is little reason to suspect that the change in wing size is due to a direct effect of nipagin on the vestigial reaction. The evidence, moreover, indicates that nipagin produces its effect by increasing the time of development. The temperature-effective period of the vestigial reaction is known to occur during a portion of the larval development. Thus, by increasing the larval period at constant temperature an effect on the vestigial wings will be produced if the duration of the vestigial reaction as compared with the rest of development is differentially affected. It appears from these results that such is the case.

In the first experiments, using varying concentrations of nipagin, it appeared that this chemical increased the time of development by decreasing the food supply. The yeast did not grow very well in the treated food although all bottles started with equal amounts of dead brewer's yeast and live yeast. The starvation experiments showed that this was the case. There was a definite increase in the time of development in wing area under both types of environmental conditions.

As stated previously, the exact relation between time of development and wing area is obscured because of the decrease in the size of the fly with increasing time of development. Under normal conditions the larvae begin to pupate at 82 hours and the last larvae pupate at 114

hours in these experiments. This variation is great because of the four-hour egg-laying periods but with even shorter egg-laying periods a spread of 18–24 hours is obtained (Powsner, 1935; Child, 1935).

This variation is a direct corollary to the nature of development which as Wright (1934) points out is the result of a large number of physical and chemical reactions, the rates and durations of which are determined by the history of the organism prior to the stage in question, correlative reactions within the organism, external environmental factors, actions of the genes within each cell, etc. In the highly heterogeneous systems of a developing larva these reactions will not go on at exactly the same rates in all organisms and there will of necessity be a spread in time of development as well as wing area under normal conditions *but no correlation between these measurements.* However, when an additional factor, lack of food, is superimposed upon this normal variation a new set of conditions prevails. The duration of the larval (feeding stage) period is lengthened, various reactions may produce minimal or even subminimal concentrations for further development and development will become somewhat disorganized. In other words, there will be a differential effect on the rates and durations of many embryological processes resulting in a modified phenotype. Under such conditions there will be a definite correlation between time of development and wing area.

With this general hypothesis in mind it is possible to postulate a number of mechanisms to account for the increased wing size. A simple scheme would allow the wing formation reactions to proceed at their normal rate but the developmental reactions which normally parallel them are slowed down, especially those reactions which determine the time at which the wing development stops. This would permit of an increased wing area. With further starvation even the wing reactions are slowed down or produce subminimal concentrations and the size of the wing decreases. This outline is, of course, very general and is not the only one which can be postulated. It merely illustrates how the general theory can be utilized.

Carry-over Effect

The carry-over effect experiments were unfortunately not extended beyond the first generation. They show, however, that there is a definite effect on the offspring of parents raised under poor food conditions—a sort of dauermodification (Jollos, 1934). It is well known that starved flies lay smaller eggs than normal ones. Powsner (1935) found that eggs laid by flies raised on poor food had a longer developmental period than eggs laid by flies raised on good food. If this delay

in development were the only factor involved a definite carry-over effect should be expected on the maternal side. In these experiments, however, there was also a paternal effect. To account for this result one must assume an effect of starvation on developing sperm. This may concern the small amount of cytoplasm carried by the sperm or perhaps a direct effect on its genic material.

The recent series of investigations at Columbia University by Rittenberg, Schoenheimer, Clarke and others in which deuterium, isotopes of nitrogen, and other elements were used to follow intermediary metabolism may bear on this problem. These workers have shown that many of the organic substances in protoplasm, even proteins, are not in a static condition. The "living proteins" are constantly interacting with their environment and may exchange their hydrogen for deuterium, and even nitrogen for one of its isotopes. Thus the composition and behavior of protoplasm is directly modified by the composition of its environment. Should the chromosomes or the genes behave in this kinetic manner of extracting substrates from the cytoplasm and releasing equivalent substances in exchange, we would have a mechanism for the production of these starvation effects and other dauermodifications, production of immunity, even differentiation during ontogeny. It is necessary, of course, to assume that the cytoplasm of the treated flies differs from that of normal cytoplasm. In this manner a modified cytoplasm may produce a change in the chromosomes. It is also of interest to note that if this is the case, we have a mechanism for an "inheritance of acquired characters," not in the old sense of the phrase but on a molecular level. This would allow the environment to produce "genetic changes" which need not be permanent. These "mutations" could return to normal in one or more generations. Plough and Ives (1935) found that variations continued to appear in generations later than those actually treated with a high temperature of 36.5° for 24 hours. These variations decreased in number in subsequent generations.

These experiments are to be continued for a number of generations. The original vestigial stock used has been discontinued in this laboratory and another isogenic stock is being prepared.

Summary

The time of development of an isogenic vestigial stock of *D. melanogaster* was increased by two methods: (1) by adding nipagin (ethyl parahydroxy benzoate) to the food, and (2) by adding only very little yeast to the food. Both methods are essentially the same in that the developing larvae are under starvation conditions. With increasing

concentration 0.05, 0.1, 0.2 and 0.4 per cent, there was an increase in the time of development and increase in the size of the wings, males showing a greater effect than females. The large wings resembled those of other vestigial alleles raised under normal conditions.

In another series of experiments 0.1 per cent nipagin and starvation were used. The larvae were removed from the culture as they pupated, to determine the relation between time of pupation and wing size. The first flies to pupate did not differ significantly in wing size from controls at that temperature. With increasing time of development there was an increase in wing size. Larvae which were very much delayed, however, developed into small flies with small wings. These wings, although small, were more differentiated and larger than the control wings.

The "carry-over" effect was studied using normal food. The treated females and males were mated with control males and females respectively. Treated males were also mated with treated females. The wings of flies from the latter mating showed the greatest carry-over effect. Treated females by control males resulted in flies having a significantly larger wing size than flies from the reciprocal cross. These results indicate that there is a definite effect on the germ cells of flies raised under starvation conditions, which effect shows itself in the subsequent development of the zygote.

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